Research Paper

Pattern recognition of lipase-catalyzed or chemically interesterified fat blends containing *n*-3 polyunsaturated fatty acids

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The feasibility to discriminate among samples of different fat blends prior and after inorganic or lipase-catalyzed interesterification, *via* pattern recognition techniques [principal component analysis (PCA) and discriminant analysis (DA)], was investigated. Blends I and II, consisting of mixtures of palm stearin, palm kernel oil and a concentrate of triacylglycerols (TAG) rich in *n*-3 polyunsaturated fatty acids (EPAX 4510TG or EPAX 2050TG) were used. These blends, prior (64 samples) and after interesterification, catalyzed by an immobilized *Thermomyces lanuginosa* lipase (Lipozyme TL IM, 54 samples) or by sodium methoxide (10 samples), were characterized by their acylglycerol profiles (25 chromatographic peaks) and solid fat content (SFC) at 10, 20, 30 and 35 °C. PCA on the multivariate data (i) showed that the initial samples were characterized by higher SFC and higher contents of high-melting TAG and (ii) suggested two separate clusters of initial and interesterified samples. DA was performed on the multivariate data to determine which of the 29 variables have discriminative power. When the 124 samples, characterized by their acylglycerols, were grouped into (i) initial and interesterified samples of blends I or II (four groups) or (ii) also by the catalyst used (six groups), 98.4% of the samples were correctly classified.

Keywords: Acylglycerol profile / Interesterification / Pattern recognition / Solid fat content / *Thermomyces lanuginosa* lipase

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1 Introduction

Interesterification (ester interchange also called transesterification) is a route to improve certain physical, sensory and nutraceutical properties of natural fats. These changes result from the rearrangement of the molecular structure of triacylglycerols (TAG), leading to a modification of the acylglycerol profile.

By interesterification among TAG, it is possible to obtain a new product with improved nutritional (digestion and adsorption), biochemical and physical properties [*e.g.* crystallization, melting point, solid fat content (SFC)], without modifying the fatty acid composition. In fact, conversely to what occurs in hydrogenation, the original content of polyunsaturated fatty acids (PUFA), namely essential fatty acids, is maintained and no formation of *trans* fatty acid isomers is observed. This is of much interest for applications in margarine, confectionary and bakery industries as well as for pharmaceutical and cosmetics purposes [1]. By mixing interesterified blends in different proportions with natural liquid oils and fats, it is possible to formulate a wide range of consumer table margarines and spreads, bakery margarines and even frying shortenings [1].

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In the food industry, the chemical interesterification reaction of fat blends is currently carried out at temperatures ranging from 50 to 120 °C, for less than 2 h, using metal alkylates or alkali metals (*e.g.* sodium methoxide and sodium) as catalyst. The interchange of the acyl groups proceeds at random. In addition, the final products may remain contaminated by residual catalyst, and the formation of considerable amounts of side products may occur, with a subsequent decrease in yield [2].

Currently, the time course of the interesterification of fat blends is indirectly assessed by the measurement for the percentage (wt-%) of the solid fraction at different temperatures, known as SFC. The knowledge of the SFC of a fat helps to decide about the suitability of a fat for further processing. The SFC values at 10, 20, 30 and 35 °C ($SFC_{10\,°C}$, $SFC_{20\,°C}$, $SFC_{30\,°C}$ and $SFC_{35\,°C}$, respectively) are related to the rheological behavior of fats at storage, packaging, bakery in winter and summer, and consumption temperatures, respectively. The $SFC_{10\,°C}$ is an indicator of the hardness of the final product at refrigerator conditions. The SFC at 35 °C is particularly important for table margarines since it is related to the extent of melting in the mouth. The $SFC_{35\,°C}$ values of the interesterified fats must be smaller than their original counterparts, and as low as possible to avoid a sandy and coarse texture of the margarine.

The growing consumer demand for natural and healthy foods has become a challenge for the food industry. In this context, the current trend includes the replacement of chemical catalysts by biocatalysts, recognized as natural, and the search for new formulations with nutraceutical properties. New products enriched in *n*-3 polyunsaturated fatty acids (*n*-3 PUFA), especially in eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3), have a great potential due to their known benefits in human health, namely the prevention and treatment of heart diseases by increasing high-density lipoprotein cholesterol levels in serum, and the reduction of inflammatory conditions [3]. Since the majority of diets do not include adequate amounts of fish rich in *n*-3 PUFA, the incorporation of these fatty acids in food products more readily available for consumption such as vegetable oils and margarines may be an option.

In the field of oils and fats, the replacement of inorganic catalysts by lipases (acylglycerol acylhydrolases, EC 3.1.1.3) has been attempted in the last years, due to the benefits of the enzymatic route relative to chemical processes [4]. Lipases are enzymes that catalyze, in aqueous media, the hydrolysis of esters, but when in organic media at low water activity, they can also catalyze esterification and interesterification reactions. In addition, the use of 1,3-selective lipases permits to maintain the fatty acids in the internal position of the acylglycerols, which is nutritionally desirable and not possible by inorganic catalysis. The 1,3-regioselectivity presented by some lipases has been explored in order to obtain, usually from low-cost raw materials, high-added-value "structured lipids" with novel important medical, nutraceutical and food applications [3]. Lipase-catalyzed interesterification of fat blends has been carried out, in batch or continuous mode, either in the presence of organic solvents or in solvent-free media [5–22].

The present work deals with chemically or lipase-catalyzed interesterified blends prepared from palm stearin, palm kernel oil and *n*-3 PUFA-rich triacylglycerols, for use in nutraceutical applications, particularly as margarine fat bases [15, 23]. Due to the high sensitivity of *n*-3 PUFA to thermal oxidation, these interesterified blends should be preferably used to be incorporated in table margarines and spreads or dressings and not used for the production of bakery margarines or frying shortenings.

The aim of this study was to evaluate the feasibility to discriminate among samples of different fat blends, prior to and after inorganic or 1,3-regioselective lipase-catalyzed interesterification in solvent-free medium, on the basis of their acylglycerol profile and their SFC values at different temperatures. It is expected that the lipase-catalyzed interesterified blends will present higher nutraceutical values than their chemically catalyzed counterparts since, due to the 1,3-regioselectivity of the lipase used, the PUFA at position 2 will be preserved.

Chemometrics, *via* pattern recognition techniques, namely principal component analysis (PCA) and discriminant analysis (DA), was the chosen tool to handle the multivariate data on acylglycerol profile and SFC values.

2 Materials and methods

2.1 Materials

Palm stearin and palm kernel oil were supplied by FIMA/VG, Produtos Alimentares, Portugal; two commercial concentrates of triacylglycerols rich in *n*-3 PUFA, "EPAX 2050TG" [20% EPA (20:5*n*-3) and 50% DHA (22:6*n*-3)] and "EPAX 4510TG" (45% EPA and 10% DHA), were a gift from EPAX AS, Lysaker, Norway. The fatty acid composition of these fats has previously been published [15]. The free fatty acid content (FFA, %) of palm stearin, palm kernel oil, and EPAX 4510T were 0.58, 0.28, and 0.28, and their peroxide values were 10, 10, and 5 meq/kg, respectively [15].

The commercial immobilized thermostable 1,3-regioselective lipase from *Thermomyces lanuginosa*, Lipozyme™ TL IM, was a gift from Novozymes A/S, Bagsvaerd, Denmark. Lipozyme™ TL IM is a food-grade granulated silica-immobilized lipase preparation adequate for the interesterification of fats in solvent-free media.

Tripalmitin, tristearin, triolein, diolein and monoolein standards were from Sigma-Aldrich, Germany; 2',7'-dichlorofluorescein standard was from Fluka; powdered sodium methoxide 95% was from Sigma-Aldrich, Germany, and silica-gel G60 thin-layer chromatography (TLC) plates were supplied by Merck. HPLC-grade acetone, from Fisher Scientific, UK, and

acetonitrile for HPLC, gradient grade, from Sigma-Aldrich, Germany, were used. Ethyl ether, *n*-hexane and glacial acetic acid p.a. were obtained from various sources.

2.2 Methods

2.2.1 Lipase-catalyzed interesterification

Interesterification reactions were performed in 100-mL thermostated cylindrical batch reactors, under magnetic stirring. The reaction media consisted of two different blend types of three fats with a total mass of 60 g: type I (palm stearin, palm kernel oil and EPAX 4510TG) and type II blends (palm stearin, palm kernel oil and EPAX 2050TG). A load of 5 wt-% of the immobilized lipase was used.

For each blend type tested, a central composite rotatable design (CCRD) corresponding to 27 experiments was performed as previously described [15] and is presented in Table 1. At the end of each experiment, 5-mL samples were taken and the biocatalyst was removed by paper filtration at approximately 70 °C. Initial and interesterified samples were stored at – 18 °C for subsequent analysis.

2.2.2 Chemical interesterification

For each type of fat blend (type I and type II containing EPAX 4510TG or EPAX 2050TG, respectively), five different samples, with the formulations used in the lipase-catalyzed experiments nos. 21–25 (Table 1), were submitted to chemical interesterification as previously described [23]. At the end of the experiments, the catalyst (powdered sodium methoxide) was removed by paper filtration at approximately 70 °C [23]. All samples (ten initial blends and ten upon chemical interesterification) were stored at – 18 °C for subsequent analysis.

2.2.3 Assay for acylglycerol profile

The changes in acylglycerol profile, occurring by the interesterification reaction, were evaluated by non-aqueous reverse-phase HPLC using a Merck Hitachi (Germany) chromatograph equipped with a reverse-phase column (100 Superspher 100-RP-18; 250 × 4 mm i.d., 5 μm particle size) and a refractive index detector. The following conditions were used: mobile phase of acetone/acetonitrile (63.5 : 36.5, vol/vol), at a flow rate of 0.8 mL/min; oven temperature of 40 °C; injection of 10 μL of a solution of the fat sample (2% wt/vol) in HPLC-grade acetone. The total HPLC run time was 35 min.

The samples (62 of initial blends, 10 samples after inorganic interesterification and 52 lipase-catalyzed interesterified samples) were assayed for their acylglycerol profile without any pretreatment.

For each blend type, up to 25 peaks corresponding to the various groups of acylglycerols were separated according to their equivalent carbon number (ECN). The ECN value is defined as [24]:

Table 1. CCRD followed in the lipase-catalyzed experiments as a function of reaction time, temperature, palm oil stearin (PS) and "EPAX 4510TG" (blend I) or "EPAX 2050TG" (blend II) concentrations used [15].

Experimental points	Experiment no.	Time [min]	Temperature [°C]	Palm stearin [%]	EPAX [%]
Factorial points	1	37.5	60	55	10
	2	37.5	60	55	20
	3	37.5	60	75	10
	4	37.5	60	75	20
	5	37.5	70	55	10
	6	37.5	70	55	20
	7	37.5	70	75	10
	8	37.5	70	75	20
	9	82.5	60	55	10
	10	82.5	60	55	20
	11	82.5	60	75	10
	12	82.5	60	75	20
	13	82.5	70	55	10
	14	82.5	70	55	20
	15	82.5	70	75	10
	16	82.5	70	75	20
Star points	17	15	65	65	15
	18	105	65	65	15
	19	60	55	65	15
	20	60	75	65	15
	21	60	65	45	15
	22	60	65	85	15
	23	60	65	65	5
	24	60	65	65	25
Center points	25	60	65	65	15
	26	60	65	65	15
	27	60	65	65	15

$$ECN = C - 2n$$

where C is the number of carbon atoms in the fatty acids chains of the acylglycerols and n is the number of double bonds in these fatty acid residues. The relative amount of each peak is given in area percentage.

A tentative identification of the various peaks separated under the HPLC conditions followed in this study was attempted (i) using a limited number of TAG standards (tripalmitin, tristearin and triolein), (ii) by comparison with chromatograms obtained with similar fats as previously reported [6, 9, 11, 25], and (iii) was based on the fatty acid composition of the individual fats used in each blend [15]. Peak identification for EPAX 4510TG and EPAX 2050TG was not possible due to the lack of standards and of available information about the TAG composition of these oils.

Also, in order to investigate whether some peaks with lower ECN were TAG or mixtures of monoacylglycerols

(MAG), diacylglycerols (DAG) and TAG with the same ECN, partial acylglycerols were separated by TLC for subsequent HPLC analysis. Samples were diluted in *n*-hexane (10%, wt/vol) and spotted on silica gel TLC plates (in continuous bands of 200 μL). Elution was carried out using a solution of *n*-hexane/ethyl ether/acetic acid (70 : 30 : 1.5, vol/vol/vol) as the mobile phase. Plates were sprayed with 2% (wt/vol) 2',7'-dichlorofluorescein in 95% ethanol (vol/vol) and observed under UV light at 366 nm. The individual bands corresponding to the various groups of compounds (MAG, DAG and TAG) were identified by comparison with standards (monoolein, diolein and triolein, respectively). MAG, DAG (the double band of 1,2- and 1,3-DAG) and TAG bands were removed separately from the plates and washed off from the silica with *n*-hexane (three times 25 mL). The solutions were recovered by paper filtration, concentrated in a rotavapor at 30 °C under reduced pressure and assayed by HPLC.

2.2.4 Data analysis

A total of 124 samples (62 initial blends, 62 interesterified blends) was characterized by (i) their acylglycerol profile (25 variables corresponding to the 25 peaks that can appear in the chromatograms) and (ii) the previously reported SFC values at 10, 20, 30 and 35 °C assayed by NMR [15, 23]. Thus, a data matrix with 124 lines (samples) and 29 rows (variables) was obtained.

PCA and DA were performed using the software Statistica™, version 6, from Statsoft, Tulsa, USA.

2.2.4.1 Principal component analysis

In this study, PCA [26–28] was carried out, aimed at (i) the reduction of the original hyperspace of the samples to a smaller-dimension space without considerable loss of information, (ii) the identification of the most important variables to characterize the different types of samples, and (iii) the identification of possible clusters of samples. By PCA, the search for eventual relationships among the acylglycerol profile and SFC values at different temperatures (10, 20, 30 and 35 °C) for the various samples before and after interesterification was also attempted.

PCA was performed on the whole matrix containing 124 samples and also on the following smaller matrices: chemical interesterification matrix containing the samples from blend types I and II, prior and after chemical interesterification (20 × 29; samples A and B); enzymatic interesterification matrix containing the initial and lipase-catalyzed samples of blend type I (with EPAX 4510TG; 52 × 29; samples C); and enzymatic interesterification matrix containing the initial and lipase-catalyzed samples of blend type II (with EPAX 2050TG; 52 × 29; samples D).

2.2.4.2 Discriminant analysis

After the clustering of samples suggested by PCA, a DA was used to determine which variables discriminate among these groups *a priori* defined and also to confirm their existence.

The basic underlying idea is to see whether groups differ with regard to the mean of a variable and then use that variable to predict group membership [27, 28]. The procedure is identical to the one-way analysis of variance or to the multivariate analysis of variance if several variables are used [28].

The models of discrimination were built step by step, and a forward stepwise analysis was performed using the following options: tolerance of 0.010; F to enter equal to 1.00 and F to remove equal to 0.00. In each step, the variable that would contribute most to the discrimination between groups was selected. The maximum number of discriminant functions (or canonical roots) will be equal to the number of groups minus one or to the number of variables in the analysis, whichever is smaller. The best combination of variables for DA includes variables that represent independent measures of product similarities and differences.

In addition, the classification functions can be used to determine to which group each case most likely belongs. The classification matrix shows the number of cases that were correctly classified and those that were misclassified.

DA was performed on the whole matrix containing 124 samples and considering subgroups of initial and interesterified samples *a priori* defined. A first DA was carried out only considering the initial and interesterified samples as a function of the blend type used, and independently of the type of catalyst used for the interesterification reaction. Thus, only four groups of samples were *a priori* defined: group of the initial samples of blend type I (A_i), group of the initial samples of blend type II (B_i), and groups of the interesterified samples of blend type I and II (A_f and B_f, respectively).

In a second DA, the effect of both fat blend type and catalyst used was considered. Therefore, the following six groups of samples were *a priori* defined: group of the initial samples of blend type I (A_i), group of the initial samples of blend type II (B_i), groups of the chemically interesterified samples of blend type I (A_f) and of blend type II (B_f), and groups of the lipase-catalyzed interesterified samples of blend type I (C_f) and of blend type II (D_f).

3 Results and discussion

3.1 Acylglycerol profiles

Each fat used in the formulations of blends I and II was first analyzed separately. Palm stearin mainly contains high-ECN peaks (Table 2: peaks 17–25; ECN 46–52) due to high amounts of palmitic (ca. 60%) and oleic (ca. 25%) acids; palm kernel oil is a lauric fat characterized by the presence of lower-ECN TAG (ECN lower than 46) due to the presence of high

Table 2. Tentative identification of the individual peaks observed in the chromatograms, as a function of their ECN and fatty acid composition (fatty acids: C, capric (10:0); Ca, caprylic (8:0); D, docosahexaenoic, DHA (22:6); E, eicosapentaenoic, EPA (20:5); L, linoleic (18:2); La, lauric (12:0); Ln, linolenic (18:3); M, myristic (14:0); O, oleic (18:1); P, palmitic (16:0); S, stearic (18:0); DAG, diacylglycerols; PS, palm stearin; PK, palm kernel oil; T_0 = initial sample; T_f = interesterified sample).

		Blend type I (PS + PK + EPAX 4510TG)		Blend type II (PS + PK + EPAX 2050TG)	
Peak no.	ECN	T_0	T_f	T_0	T_f
1	?	EPAX + DAG	EPAX + DAG	DAG	DAG
2	28	LaCaCa + DAG	LaCaCa + DAG + EDCa	EPAX/LaCaCa + DAG	EPAX/LaCaCa + DAG+ EDCa
3	28	CCCa + DAG	CCCa + DAG	CCCa + DAG	CCCa + DAG
4	30	CCC + DAG + EEE, DDD, EED, DDE	CCC + DAG + EEE + DDD + EED + DDE EEC + (C22:5) ECa	EEE + DDD + EED + DDE CCC + DAG	CCC + DAG + EEE + DDD + EED + DDE + EEC + (C20:4) DCa+ (C22:5) ECa
5	32	LaCC + CaLaLa	DAG + LaCC + CaLaLa + LnDC	LaCC + CaLaLa + EPAX	LaCC + CaLaLa + EPAX + DAG + LnDC
6	34	LaLaC + CLaM	DAG + LaLaC + LDC + LnLaE	LaLaC	LaLaC + DAG + LDC+ LnLaE
7	36	LaLaLa	DAG + LaLaLa + DOC + ELLa	LaLaLa	LaLaLa + DAG + DOC + ELLa
8	38	LaLaM	LaLaM + EOLa + PLaD + SDC + MMD	LaLaM	LaLaM + EOLa + PLaD + SDC + MMD
9	40	LaLaO	LaLaO + LaES	LaLaO	LaLaO + LaES
10	40	LaMM	LaMM + PEM	LaMM	LaMM + PEM
11	42	LaOM	LaOM	LaOM	LaOM
12	42	LaPM	LaPM	LaPM	LaPM
13	44	LaOO	LaOO	LaOO	LaOO
14	44	LaOP	LaOP	LaOP	LaOP
15	44	LaPP + MOM	LaPP + MOM	LaPP + MOM	LaPP + MOM
16	46	MOO + OOL + PLO	MOO + OOL + PLO	MOO + OOL + PLO	MOO + OOL + PLO
17	46	MOP + PLP	MOP + PLP	MOP + PLP	MOP + PLP
18	48	OOO	OOO	OOO	OOO
19	48	POO	POO	POO	POO
20	48	POP	POP	POP	POP
21	48	PPP	PPP	PPP	PPP
22	50	OOS	OOS	OOS	OOS
23	50	POS	POS	POS	POS
24	50	PPS	PPS	PPS	PPS
25	52	SOS	SOS	SOS	SOS

amounts of short- and medium-chain saturated fatty acids (ca. 44% lauric and 15% myristic acids) and also about 10% palmitic acid. The concentrates EPAX 4510TG and EPAX 2050TG have the lowest-ECN species due to the presence of long-chain polyunsaturated fatty acids: 63 and 88% of *n*-3 PUFA from a total of 67 and 91% PUFA content, respectively [15]. Thus, the content of Table 1 is a feasible approach based on the original composition of the fats and oils used and on the predictable formation of new acylglycerols. Only the use of other powerful analytical techniques such as mass spectroscopy could allow a correct identification of the 25 peaks appearing in the initial and interesterified fat blends.

As an example, Fig. 1 shows the chromatograms of (i) the initial sample of blend type I corresponding to the center point of the experimental design followed (65% palm stearin + 20% palm kernel oil + 15% EPAX 4510TG; Table 1) and (ii) of the same sample after enzymatic (at 65 °C for 60 min) or (iii) chemical interesterification. For both fat blends used, noticeable modifications on their acylglycerol profiles were observed upon either inorganic or lipase-catalyzed interesterification reaction (Fig. 1).

In the vast majority of samples, it was observed that a decrease in the amounts of peaks with high ECN (*e.g.* peaks 20 and 21, ECN = 48), which are present in large amounts in palm stearin, is accompanied by an increase of lower-ECN peaks [*e.g.* peaks 6 (ECN = 34), 14 (ECN = 44) and 15 (ECN = 44)]. The decrease in ECN values for the interesterified TAG is likely a result of the increased unsaturation of these TAG compared to palm stearin and palm kernel oil TAG. In the lipase-catalyzed interesterified samples, a significant increase in peak 2 (ECN = 28) was observed for both fat blends used.

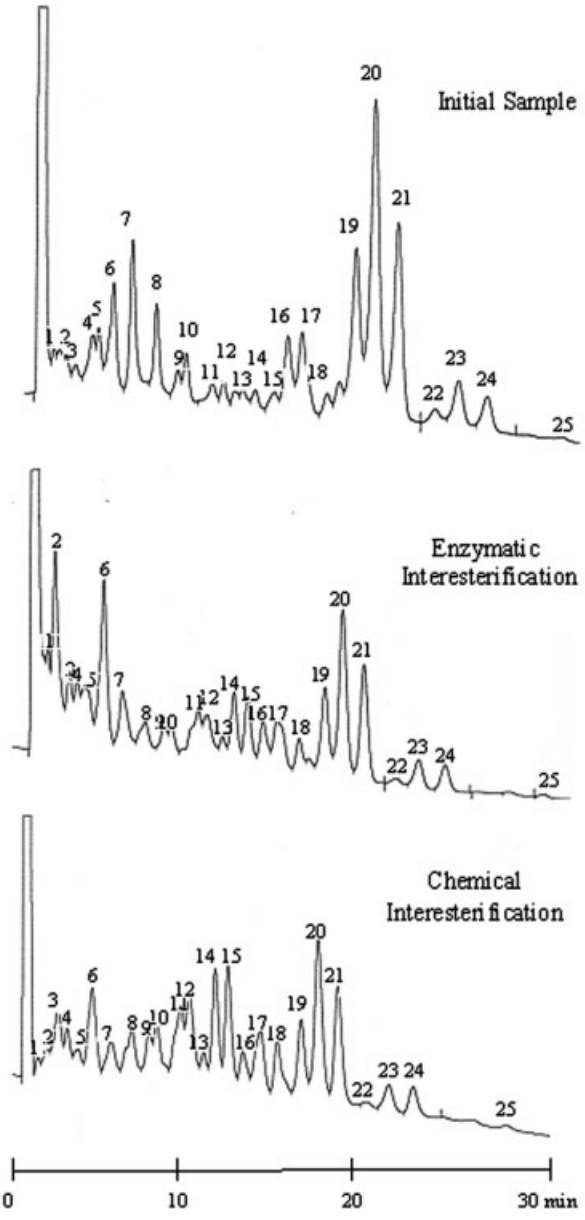

Figure 1. HPLC chromatograms of the acylglycerol profiles of the initial blend sample containing 65% palm stearin, 20% palm kernel oil and 15% EPAX 4510TG (blend I), and upon interesterification catalyzed by Lipozyme TL IM, for 60 min at 65 °C, or chemical interesterification.

The consumption of TAG species with high ECN, accompanied by the increase of acylglycerol species of lower ECN, has also been observed (i) during the 1,3-selective lipase-catalyzed interesterification of fat blends of palm stearin with coconut oil [11], palm kernel oil [16] and canola oil [29], and (ii) during the chemical interesterification of ternary blends of palm, palm kernel and sunflower oils [25] and palm stearin, palm kernel and sunflower oils [30].

Since species of low ECN may be TAG or partial acylglycerols with the same ECN, the initial samples corresponding to the center points of the experimental designs (Table 1) and these samples after enzymatic interesterification were submitted to TLC analysis, both for fat blends I and II. The TLC analysis showed an increase of the DAG bands (visual observation) in the interesterified samples, while the presence of MAG was not detected. The double bands corresponding to 1,3- and 1,2-DAG and the TAG band were analyzed by HPLC (c.f. 2.2.3.). The chromatograms obtained for the various fractions confirm the presence of DAG together with TAG with the same ECN, either in the initial or in the interesterified samples (Fig. 2).

The increase of DAG levels, by lipase-catalyzed interesterification of palm stearin with palm kernel oil [9], palm stearin and coconut oil [11, 16], flaxseed oil with palm olein or palm stearin [13], palm stearin with canola oil [29], and olive oil with fully hydrogenated palm oil [21], was also reported.

3.2 Principal component analysis

PCA was performed on the data concerning acylglycerol composition and SFC values of both the initial fat blend samples and upon chemical or lipase-catalyzed interesterification.

As referred earlier, the description of the interesterification experiments and the SFC values of these samples have been published previously [15, 23]. In all the experiments, the interesterification catalyzed either by Lipozyme™ TL IM or by sodium methoxide promoted a decrease in the SFC values of the fat blends at 10, 20, 30 and 35 °C. The variation ranges for the SFC values assayed at each test temperature were similar for both blend types used: $SFC_{10\,°C}$ varied from 49 to 68, $SFC_{20\,°C}$ varied from 28 to 57, $SFC_{30\,°C}$ ranged from 15 to 40, and the $SFC_{35\,°C}$ values from 11 to 33 [15, 23]. The highest SFC values correspond to the samples containing higher amounts of palm stearin. Upon interesterification, a decrease in all the SFC values was observed. The SFC variation ranges for the interesterified samples were similar for both blend types, whatever the catalyst type used: $SFC_{10\,°C}$ varied from 28 to 60, $SFC_{20\,°C}$ varied from 19 to 39, $SFC_{30\,°C}$ varied from 7 to 24, and the $SFC_{35\,°C}$ values from 0.5 to 18 [15, 23].

The sensory attribute of "coolness" is connected with the content in saturated TAG. The SFC values at 30 and 35 °C are principally determined by this content [1]. At consumption of a margarine, the sensation of "coolness" and the melting speed have both been reported to increase with the difference between the $SFC_{20\,°C}$ and the $SFC_{30\,°C}$ values of the interesterified fat blend, as referred by G. Langsbergen (at http://www.fatsforfoods.com, 2008). The values for ($SFC_{20\,°C}$ – $SFC_{30\,°C}$) obtained for the interesterified blends, containing either EPAX 4510TG (blends I) or EPAX 2050TG concentrates (blends II), varied between 10 and 19. At 25 wt-% of both types of EPAX, the values of ($SFC_{20\,°C}$ – $SFC_{30\,°C}$) of the interesterified blends still fulfill the technological requirements for the production of table margarines [15].

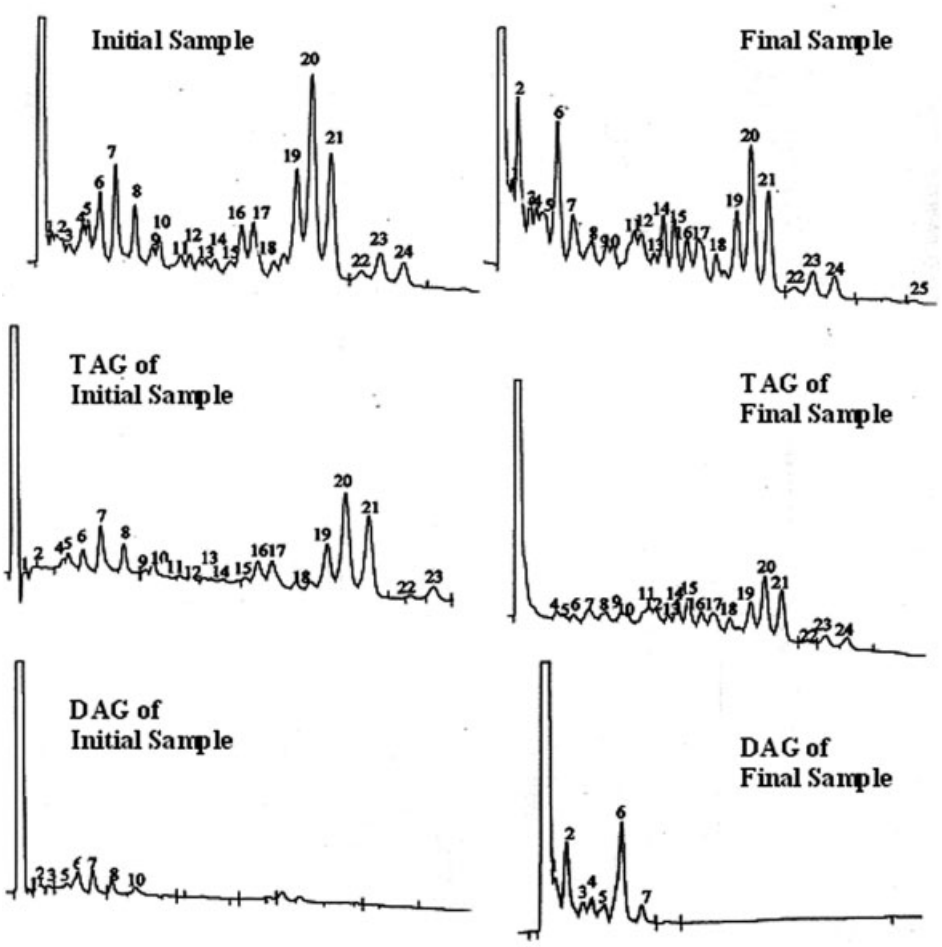

Figure 2. Acylglycerol profiles of the whole sample of Fig. 1, prior to and after lipase-catalyzed interesterification, and the chromatograms of the respective TAG and DAG fractions, separated by TLC.

The results concerning PCA of the various data matrices are presented in Table 3. The initial 29-dimensional hyperspace can be highly reduced to a 7-dimensional space, for the chemical matrix, or to 6-dimensional spaces (eigenvalues greater than 1) for both enzymatic matrices, which account for more than 90, 85 and 84% of the original information (variance), respectively.

When all the initial and interesterified samples (chemical and enzymatic matrices) are analyzed together, the original information can be visualized in a 6-dimension space, explaining 77% of the information contained in the original data (data not shown).

Figure 3 is a plot in the reduced space, defined by the first and the second significant principal components, of the samples of both blend types, prior and after lipase-catalyzed interesterification, and variable loadings (*i.e.* the correlations between the original variables and the principal components).

For every matrix, the first principal component (Factor 1, F_1) is positively correlated with medium- and low-ECN peaks and negatively correlated with the variables corresponding to the acylglycerols with higher ECN (46, 48 and 50) and the SFC values at 10, 20, 30 and 35 °C. As previously reported, a decrease in all SFC blend values was observed upon lipase-catalyzed as well as upon chemical interesterification [15, 23]. In fact, high SFC values are ascribed to the presence of TAG with long-chain saturated fatty acids, *i.e.* of high ECN.

When the samples were plotted on the plane formed by the first and the second principal components, F_1F_2 (Fig. 3), and also on the plane F_1F_3 for the chemical matrix (figure not shown), two clusters become evident: a cluster of initial samples, defined by higher SFC values and higher amounts of TAG of high ECN, and a cluster of interesterified samples, showing increased amounts of lower-ECN acylglycerol species and lower SFC values. A translation of the initial group of samples from the left to the right side of the graphs is observed upon interesterification, regardless of the interesterification method and of the initial blend composition used. These projections are in accordance with the analysis of the chromatograms and of the SFC values of these samples [15, 23]. In fact, a decrease of TAG of ECN 46–50 is observed, either by chemical or enzymatic interesterification. This decrease is accompanied by an increase in the amount of acylglycerols

Table 3. PCA eigenvalues and variances explained by the first 20 principal components extracted from the various data matrices of samples defined by the acylglycerol profile and SFC values ($SFC_{10\,°C}$, $SFC_{20\,°C}$, $SFC_{30\,°C}$ and $SFC_{35\,°C}$), prior to and upon chemical or enzymatic interesterification.

	Chemical interesterification matrix			Enzymatic interesterification matrices					
	Blend types I and II			Blend type I (EPAX 4510TG)			Blend type II (EPAX 2050TG)		
Principal component	Eigenvalue	Variance [%]	Cumulative variance [%]	Eigenvalue	Variance [%]	Cumulative variance [%]	Eigenvalue	Variance [%]	Cumulative variance [%]
1	13.25	45.70	45.70	15.08	52.00	52.00	12.99	44.78	44.78
2	3.51	12.10	57.80	3.99	13.74	65.74	3.34	11.50	56.28
3	2.57	8.86	66.66	2.39	8.24	73.98	3.23	11.14	67.42
4	2.23	7.69	74.34	1.28	4.42	78.40	2.55	8.80	76.22
5	1.83	6.31	80.65	1.17	4.05	82.45	1.36	4.70	80.92
6	1.59	5.48	86.13	1.00	3.44	85.89	1.04	3.57	84.49
7	1.35	4.66	90.78	0.79	2.73	88.62	0.77	2.65	87.14
8	0.86	2.96	93.74	0.78	2.68	91.31	0.66	2.28	89.42
9	0.63	2.16	95.90	0.64	2.22	93.53	0.65	2.23	91.65
10	0.37	1.28	97.18	0.51	1.76	95.29	0.41	1.40	93.05
11	0.31	1.07	98.25	0.28	0.98	96.26	0.40	1.37	94.42
12	0.20	0.70	98.95	0.23	0.80	97.07	0.29	1.01	95.43
13	0.11	0.38	99.33	0.22	0.76	97.82	0.25	0.86	96.29
14	0.09	0.30	99.63	0.15	0.53	98.35	0.23	0.79	97.08
15	0.04	0.14	99.77	0.11	0.39	98.74	0.21	0.73	97.81
16	0.03	0.09	99.86	0.08	0.29	99.03	0.16	0.54	98.35
17	0.02	0.06	99.92	0.08	0.26	99.28	0.12	0.42	98.76
18	0.01	0.05	99.97	0.06	0.21	99.50	0.12	0.41	99.17
19	0.01	0.03	100.00	0.05	0.16	99.66	0.07	0.26	99.43
20	0.00	0.00	100.00	0.03	0.10	99.75	0.05	0.18	99.61

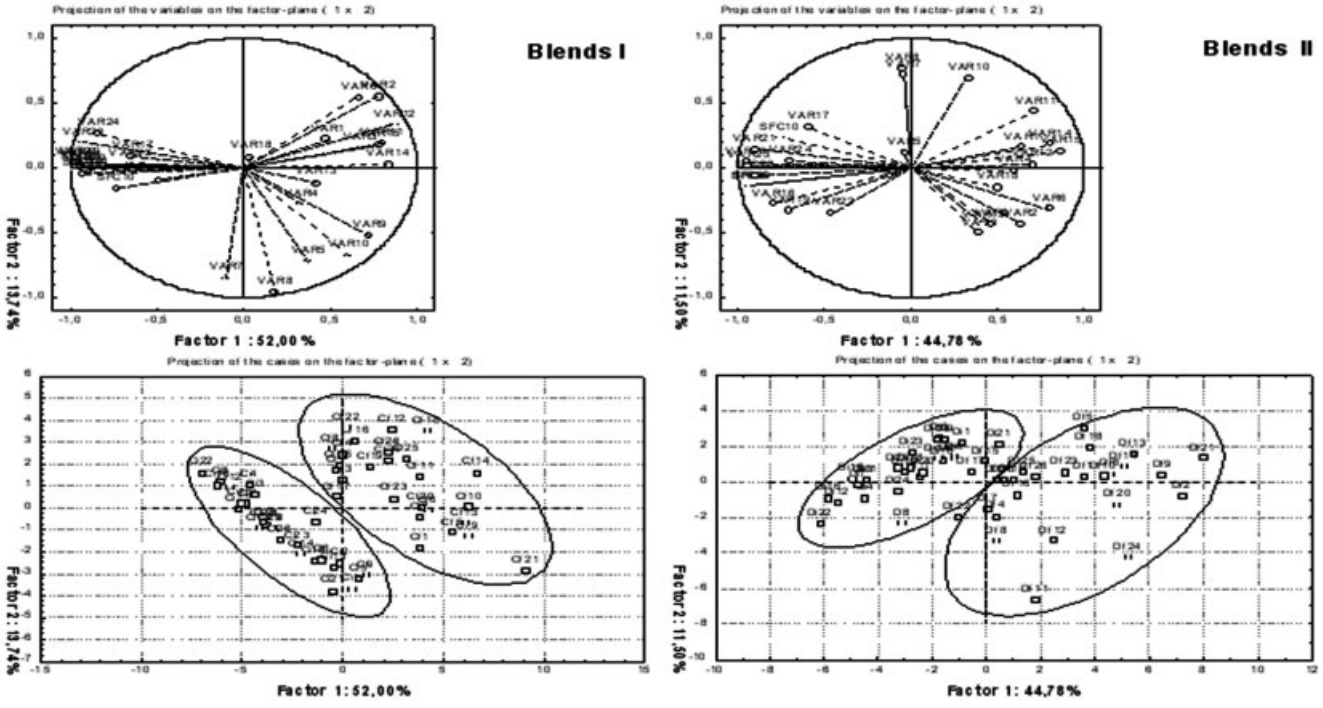

Figure 3. PCA projections of all the variables (VAR1–VAR25: chromatographic peaks; $SFC_{10\,°C}$, $SFC_{20\,°C}$, $SFC_{30\,°C}$ and $SFC_{35\,°C}$) and of (Blends I) the initial samples, C_i, of blends type I and upon lipase-catalyzed interesterification (C_f) and (Blends II) of the initial samples D_i of blends type II and upon lipase-catalyzed interesterification (D_f), on the plane F_1F_2 defined by the first and the second principal components.

and/or the formation of new acylglycerols of lower ECN (28–44). Some of these new species are DAG (peaks 1–7; ECN equal or lower than 36), as confirmed by TLC.

When the 124 samples were analyzed together, the projection of such a large number of samples does not allow for a clear identification of separate groups (figure not shown).

3.3 Discriminant analysis

The PCA of the various samples suggested the presence of groups of samples in the original hyperspace containing these samples. Thus, DA was performed on the original data in order to (i) assess the existence of the clusters suggested by PCA and (ii) to identify the variables that in fact discriminate among these groups *a priori* defined. In both DA performed, samples were characterized (i) only by their acylglycerol profile, (ii) only by their SFC values, and also (iii) by the acylglycerol profile together with their SFC values.

Considering the DA as a function of the blend type used, 122 of the 124 samples (98.4%) defined only by their acylglycerol profile were correctly classified (Table 4). The corresponding classification functions (not shown) excluded the following five peaks of the chromatograms since they showed no discriminant power among sample groups: peak 9 (LaLaO), peak 15 (LaPP + MOM), peak 19 (POO), peak 20 (POP) and peak 24 (PPS). It is worthy of notice that when the samples were characterized only by their SFC values, just about 51% of the samples were correctly classified (data not shown).

When the SFC values and the acylglycerol profiles were used simultaneously to characterize the samples, the SFC values showed to discriminate among the sample groups. However, the same percentage of correctly classified samples (98.4%), as obtained if only the acylglycerol profiles were considered, was achieved (data not shown), indicating that for sample characterization, the acylglycerol profile is sufficient. This may be explained by (i) a relatively low discriminant power of the SFC values and (ii) by a weak relationship between the SFC values and the acylglycerol profiles.

With respect to the DA as a function of both blend and catalyst types (six groups *a priori* defined), the results obtained in terms of percentage of samples correctly classified were similar to those obtained in the previous DA where only four groups were *a priori* defined (98.4%), either when only the acylglycerol profiles or when the SFC values were considered together with the acylglycerol profiles (Tables 4, 5). To discriminate among the six groups, all the peaks except peak 24 of the chromatogram, corresponding to the TAG PPS, have to be considered. Thus, the peaks 9 (LaLaO), 15 (LaPP + MOM), 19 (POO) and 20 (POP) must be included to be able to discriminate among enzymatically and chemically interesterified samples.

Interestingly, except for peak 9, all these additional TAG present oleic acid at the *sn*-2 position, which will be not available for the action of the *sn*-1,3-selective *Thermomyces lanuginosa* lipase. Thus, the 1,3-regioselectivity of this lipase is confirmed by the acylglycerol profiles of the lipase-catalyzed interesterified samples. Also, peaks 9 and 15 correspond to TAG containing lauric acid at external positions. A high affinity of Lipozyme TL IM towards lauroyl residues was also observed in the production of structured TAG containing conjugated linoleic acid at the *sn*-2 position and lauric acid at external positions, in solvent-free media [22].

With regard to the SFC values, all of them showed discriminant power among the six groups of samples. However, only 51.6% of the samples were correctly classified into the six groups *a priori* defined (data not shown). Again, when a DA was performed considering the samples defined both by their acylglycerol profile and SFC values, the classification prediction was not improved.

Figures 4 and 5 show the projections of the samples, characterized by the acylglycerol profiles, on the planes defined by the canonical roots 1 and 2 and 1 and 3, respectively when four or six groups were *a priori* defined. In both

Table 4. DA classification matrix for the groups of initial and interesterified samples defined by the blend type used (samples A: blend I with EPAX 4510TG; samples B: blend II with EPAX 2050TG) before (A_i and B_i) and after (A_f and B_f) interesterification (chemical or enzymatic). Samples were characterized by their acylglycerol profiles.

	[%]	Columns: Predicted classifications			
Group	Correct	A_i	A_f	B_i	Bf
A_i	100.0	31	0	0	0
A_f	96.8	0	30	0	1
B_i	96.8	0	0	30	1
B_f	100.0	0	0	0	31
Total	98.4	31	30	30	33

Table 5. DA classification matrix for the six groups of samples defined by the blend type (samples A and C: blend I with EPAX 4510TG; samples B and D: blend II with EPAX 2050TG) and catalyst type used. Samples prior to (A_i and B_i,) and upon interesterification (chemical: A_f and B_f; enzymatic: C_f and D_f) were characterized by their acylglycerol profiles.

	[%]	Columns: Predicted classifications					
Group	Correct	A_i	A_f	B_i	B_f	C_f	D_f
A_i	100.0	31	0	0	0	0	0
A_f	100.0	0	5	0	0	0	0
B_i	96.8	0	0	30	0	0	1
B_f	100.0	0	0	0	5	0	0
C_f	96.2	0	0	0	0	25	1
D_f	100.0	0	0	0	0	0	26
Total	98.4	31	5	30	5	25	28

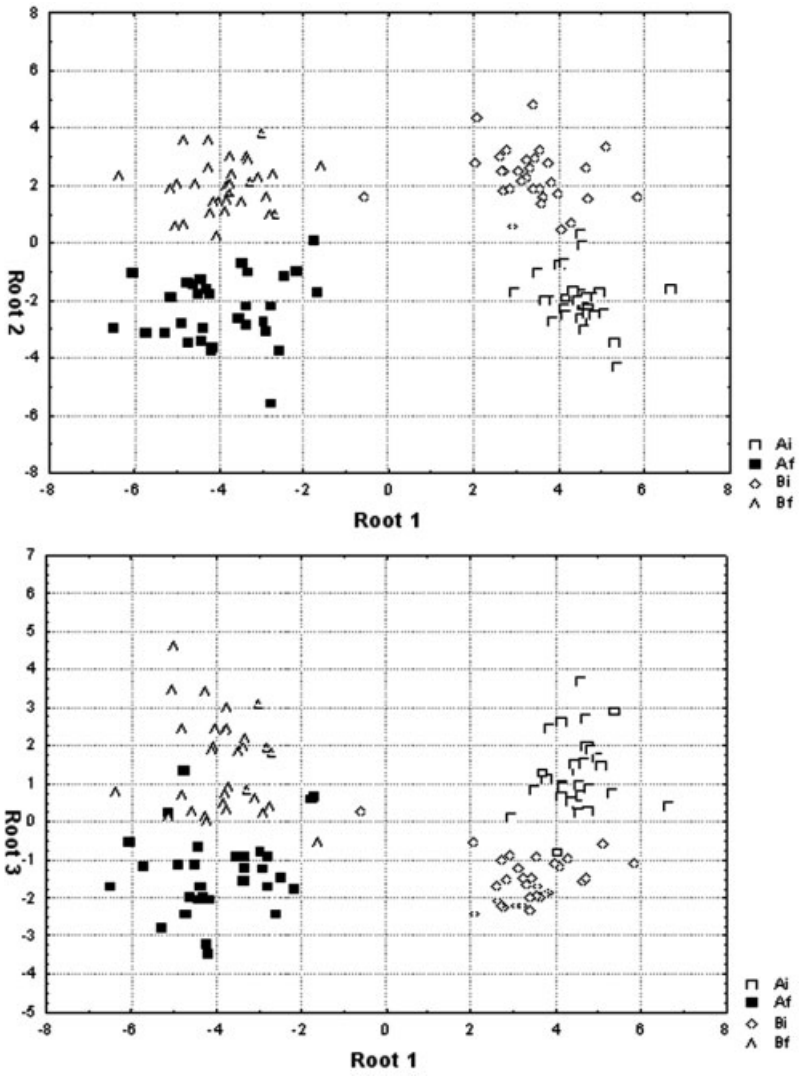

Figure 4. DA-plot of the groups of initial samples of blends I (A_i) and II (B_i) and upon interesterification (A_f and B_f), on the planes defined by the canonical roots 1 and 2, and 1 and 3. Samples were defined by all the variables.

cases, the clustering of the samples into the groups previously defined is well illustrated. Similarly to the PCA plots, a translation of the groups of the initial samples is observed upon interesterification.

4 Conclusions

The obtained results showed that PCA is a useful exploratory tool that can be used to study lipase-catalyzed and chemical interesterification of fat blends, in order to give global information on the changes occurring in the acylglycerol composition and SFC values.

The final SFC values at the different temperatures of the interesterified fat blends indicate that these blends can be used to prepare typical base stocks for margarine blends enriched with *n*-3 PUFA [15]. The margarine manufacturer will choose the correct proportions of natural liquid oils and fats and interesterified basestock, which will be dictated by the SFC values of the available interesterified fat blends, to formulate the requested type of margarine.

The DA allows for the correct identification of samples *via* the knowledge of their acylglycerol profiles. In fact, the knowledge of the acylglycerol profile of an unknown fat blend sample is enough (i) to predict the blend type used in its formulation, (ii) to know if the sample has been previously interesterified or not, and (iii) in the case of an interesterified sample allows predicting the type of catalyst used. Also, DA was able to confirm the 1,3-regioselectivity of Lipozyme TL IM solely on the basis of the acylglycerol profiles of the interesterified samples.

The SFC values on their own are not sufficient to correctly predict the group to which a sample most likely belongs. When considered together with the acylglycerol profile, the

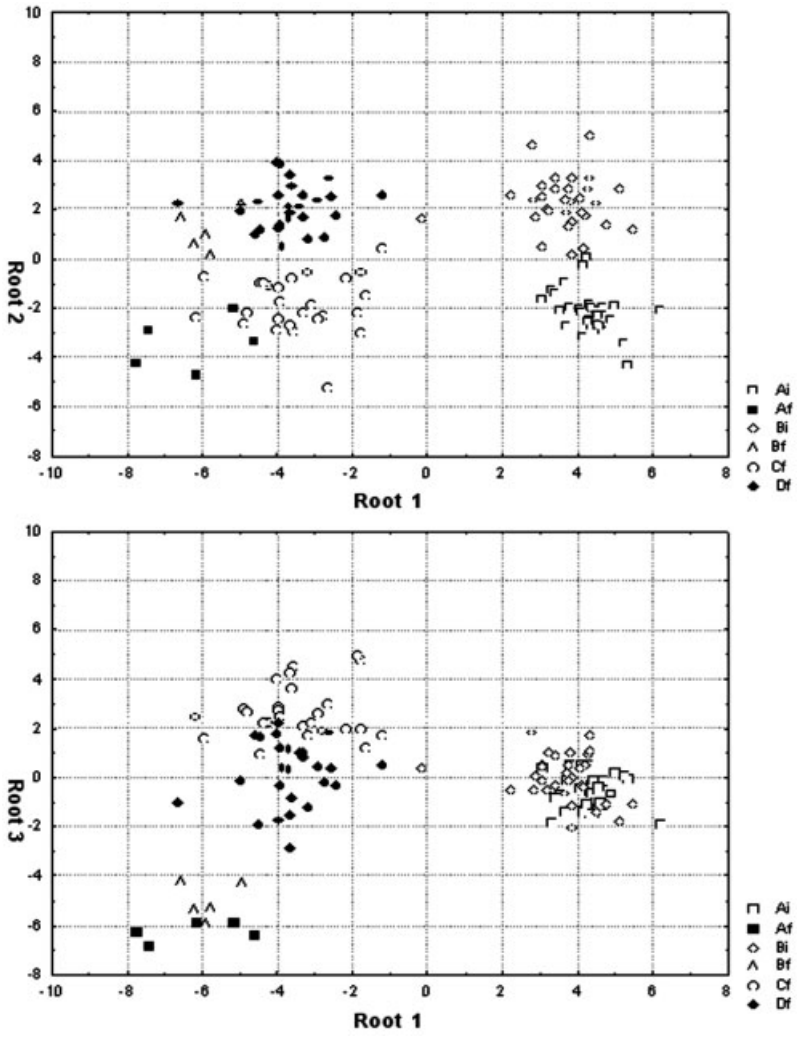

Figure 5. DA-plot of the groups of initial samples of blends I (A_i) and II (B_i) and after chemical (A_f and B_f) or lipase-catalyzed interesterification (blend I: C_f; blend II: D_f) on the planes defined by the canonical roots 1 and 2, and 1 and 3. Samples were defined by all the variables.

SFC values did not increase the level of correct classification prediction.

The aim of this work was thus accomplished, *i.e.* both chemically and lipase-catalyzed interesterified samples could be distinguished from native oils and their blends by applying PCA and DA to simple HPLC chromatograms, overcoming the use of expensive equipment for the identification of individual compounds. In addition, this study indicates that chemometrics is an extremely useful tool to predict results regarding the interesterification of fat blends.

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Conflict of interest statement

The authors have declared no conflict of interest.

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